Proc. Natl. Acad. Sci. USA
Vol. 78, No. 7, pp. 4236–4240, July 1981
Biochemistry

Complete amino acid sequence of a human pituitary glycopeptide: An important maturation product of pro-opiomelanocortin

(pituitary hormones/corticotropin/lipotropin/sequence microdeterminations/aldosterone-stimulating substance)

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Communicated by Elwood V. Jensen, April 9, 1981

ABSTRACT **A glycopeptide isolated in relatively large amounts from human pituitary glands was completely purified, and its sequence was determined. The primary sequence represents the NH_2-terminal 76 amino acid residues of pro-opiomelanocortin (POMC). This important secretory product of POMC was shown to possess an interesting aldosterone-stimulating activity on a human adrenal aldosteronoma. It is *O*-glycosylated at Thr-45 and *N*-glycosylated at Asn-65. Only one sequence variation with the human genomic DNA was found. Furthermore, comparison with the other preferred cleavage sites of human POMC reveals that the pair of basic residues Lys-Arg represents the major sites of enzymatic maturation of this precursor molecule. This predicts a highly specific type of enzyme involved in the maturation of POMC in the anterior lobe of the human pituitary.**

That the pituitary polypeptides corticotropin (ACTH; adrenocorticotropic hormone) and β-lipotropin (β-LPH; β-lipotropic hormone) originate from a common pluripotent precursor molecule called pro-opiomelanocortin (POMC) (1) has now been well-established in a number of mammalian species (1–7). After excision of the 26-residue signal peptide (8–10), pulse–chase studies have shown the maturation of POMC into β-LPH/β-endorphin, ACTH/α-melanotropin (MSH; melanocyte-stimulating hormone), and a stable NH_2-terminal glycopeptide (4–14). The advent of recombinant DNA techniques has greatly facilitated studies of the structure of genes coding for protein precursors. By using such techniques, the mRNA sequence of bovine pars intermedia pre-pro-opiomelanocortin was first determined (15). Subsequently, the genomic DNA sequences of human (16), bovine (17), and rat (18) homologues were reported. From the nucleotide sequences, it became apparent that the NH_2-terminal segment of POMC contained a MSH sequence homologous to α- and β-MSH, and it was called γ-MSH (15). Pituitary protein purification and characterization allowed the demonstration of the existence of such an NH_2-terminal glycopeptide in both human (19–21) and porcine (22, 23) pituitaries. Because these and pulse–chase studies used NH_2-terminal sequence analysis and tryptic-peptide mapping characterizations, the exact COOH-terminal length of the isolated glycopeptide is not known with certainty. Furthermore, the possible presence of one or two glycosylation sites within this segment has been suggested (7, 10, 12–14, 20, 23) but not determined.

In order to ascertain the exact COOH-terminal length and number of glycosylation sites of the isolated human NH_2-terminal glycopeptide (19, 20), the complete amino acid sequence was determined. The data reported here show that this human NH_2-terminal glycopeptide is an important product of maturation in the pituitary gland and that it is composed of 76 amino acid residues. Two glycosylation sites were found. One is an *N*-glycosidic chain at Asn-65 and the other is possibly an *O*-glycosidic site at Thr-45. Compared to the human genomic DNA sequence reported (16), only one variation has been reconfirmed—namely, the presence of Arg-22 (20) instead of Gly-22 (16).

MATERIALS AND METHODS

Isolation of Human NH_2-Terminal Peptide. The isolation method and the purification by high-performance liquid chromatography (HPLC) have been reported (20).

Amino Acid and Sequence Analyses. For amino acid analysis, the native peptide was first reduced and carboxymethylated (20). Triplicate analysis of the 5.7 M HCl hydrolysates (for 24, 48, and 72 hr of hydrolysis) of the human NH_2-terminal peptide was performed with an updated Beckman 120C amino acid analyzer modified to allow up to 0.5-nmol-detection sensitivity. Determinations of the glucosamine (GlcN) and galactosamine (GalN) values were not corrected for hydrolysis loss at 24 hr. For the cyanogen bromide peptides, only 24-hr hydrolysates were analyzed. Automatic sequence analysis was carried out by using 3.30 mg of Polybrene (Aldrich) as carrier on a Beckman 890B sequenator equipped with a cold trap (1, 5, 6, 8–13, 20). This instrument was also equipped with a Sequemat P6 auto converter, and the phenylthiohydantoin conversion products, in 1.5 M HCl/MeOH, were analyzed by HPLC with an Altex 5-μm ODS column and a tetrahydrofuran/acetonitrile/Na acetate gradient (unpublished data). The HPLC instrument consisted of two Waters Associates 6000A pumps equipped with a Wisp auto injector, a Waters Associates Data Module plotter/integrator, and a model 450 variable wave length UV/visible detector (used at 269 nm).

Cyanogen Bromide Cleavage and Peptide Purification. Cyanogen bromide cleavage was carried out by starting with 1.4 mg of human NH_2-terminal peptide and using 70% (vol/vol) formic acid and a 1000:1 mole ratio of CNBr to peptide (assuming a molecular weight of about 13,000). The reaction was allowed to proceed for 24 hr in the dark, and the product was then lyophylized three times.

Purification of the CNBr fragments that were produced was done on a Waters Associates μ-C18 column (0.39 × 30 cm) eluted with a linear gradient of 0.02 M triethylamine phosphate (pH 3.0) and 2-propanol (20, 24) at 210 nm. The collected purified peptides were lyophylized, hydrolyzed for 24 hr, and subjected to amino acid analysis. This allowed the separation of three major peptides denoted "CNBr I, II, and III" (see Table 2). The GlcN and GalN values were not corrected for hydrolysis loss.

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Abbreviations: POMC, pro-opiomelanocortin; ACTH, corticotropin (adrenocorticotropic hormone); β-LPH, β-lipotropin (β-lipotropic hormone); MSH, melanotropin (melanocyte-stimulating hormone); HPLC, high-performance liquid chromatography.

RESULTS

Isolation of a Human Glycopeptide Containing the NH_2-Terminal Sequence of Pro-Opiomelanocortin. As reported (19, 20), a homogenous preparation of a human glycopeptide from an HCl/acetone extract of frozen human pituitary glands was obtained after carboxymethylcellulose and Sephadex G-75 chromatography and HPLC. This methodology allowed the purification of about 15 mg of this material per 250 human glands. Upon reduction and radiolabeling of the cysteine residues with [^{14}C]iodoacetamide, a sequence of Cys-2, -8, -20, and -24 was determined (19, 20). These results also showed that about 30% of the molecules lacked the first residue, giving a partial sequence of Cys-1, -7, -19, and -23 (20). An identical partial cysteine sequence is found at the *N*-terminus of POMC in rat (8, 13, 18), mouse (10, 25), bovine (15), and porcine (22, 23) homologues, emphasizing the sequence conservation of these residues. Furthermore, no labeling of this human peptide was observed if the reduction step was omitted. This result shows that the four cysteine residues are involved in disulfide bridges (24).

The amino acid analyses of the reduced and carboxymethylated human peptide after triplicate 24-, 48-, and 72-hr hydrolyses (Table 1) showed that the predicted length of the peptide is 76 amino acid residues. This is to be contrasted with the previously reported length of 103 (20). However, the apparent molecular weight of this peptide in the presence of $NaDodSO_4$ was found to be ≈18,000 (20). Such a large molecular weight would not be expected for a 76-residue peptide. It is now clear that this peptide is glycosylated (Table 1), and glycopeptides are known to migrate anomalously in the presence of $NaDodSO_4$, giving the impression of higher molecular weights (26). Uncertainty in estimating the true molecular weight of this peptide has been reported also for the mouse homologue, a 16,000 molecular weight fragment (7). Upon gel filtration under denaturing conditions, the mouse homologue gave a molecular weight of 11,200 ± 500 (7). In Table 1, the recently reported amino acid composition of a similar human peptide, denoted "DEAE II" (21) is also compared with that obtained in this study. Because of the variations observed, it was imperative to determine the exact and complete amino acid sequence of the purified human peptide.

Table 1. Human NH_2-terminal peptide

Amino Acid	24 hr	48 hr	72 hr	Integer	DEAE II (ref. 21)
Asx	8.07	8.21	7.95	8	8.7
Thr	3.73	3.54	3.50	4*	4.1
Ser	9.10	8.56	8.16	10*	7.6
Glx	10.16	9.79	9.68	10	9.8
Pro	5.04	4.72	5.25	5	5.0
Gly	6.90	7.31	7.20	7	8.0
Ala	3.03	3.21	3.10	3	5.3
Cys	3.20	3.64	3.54	4	3.9
Val	1.33	0.90	1.05	1	3.2
Met	1.78	1.60	1.07	2*	2.1
Ile	0.70	0.95	0.90	1	1.3
Leu	6.00	6.10	6.10	6	6.4
Tyr	0.75	1.01	1.02	1	1.1
Phe	2.50	2.63	2.85	3	3.0
His	1.12	0.98	1.00	1	1.8
Lys	2.28	2.36	2.38	2	4.1
Trp	ND	ND	ND	2†	1.6
Arg	5.98	6.36	6.05	6	5.0
GlcN	+++	—	—	+++	—
GalN	+	—	—	+	—
Total	—	—	—	76	≈82

Amino acid analysis after each of three acid hydrolyses (24, 48, and 72 hr) of the isolated, reduced, and carboxymethylated human NH_2-terminal peptide. Comparison with the recently reported composition of a similar human peptide (21) is also shown. The presence of GlcN and GalN was apparent at 24 hr, but because of destruction at 48 and 72 hr of hydrolysis, it was rarely seen. ND, not determined due to destruction upon hydrolysis with 5.7 M HCl.

* Corrected for destruction during hydrolysis (10–15%).

† Confirmed by sequence and by hydrolysis using 4 M methanesulfonic acid (20).

NH_2-Terminal Amino Acid Sequence. One milligram of reduced and carboxymethylated human NH_2-terminal peptide was subjected to 60 cycles of Edman degradation. The analysis of phenylthiohydantoin derivatives collected at each cycle was done by HPLC, and this allowed the positive identification of the first 49 residues (24). This sequence confirms the previously reported one and extends it to residue 49 (20). Comparison with that expected from the genomic DNA (ref. 16; Fig. 1) showed that a glutamic acid residue is at position 19, at the reported intron site. Although this could have been predicted from the "G-A-G" sequence observed (16), the presence of Ala-19 in bovine (15), porcine (22–23), and rat (8, 18) homologues posed a doubt in assigning this Glu-19 in the nucleotide sequence (16). In the sequence reported here, this residue is confirmed (20). However, as reported previously (20), the Gly-22 obtained from the genomic DNA study (16) is not seen in our sequence, but rather is replaced by Arg-22. This residue seems to be invariant in all homologous proteins sequenced so far (8, 22, 23). Therefore, the reason for the difference in sequence between protein and genomic DNA is yet to be adequately resolved.

Cyanogen Bromide Cleavage. The presence of two methionine residues in the molecule (Table 1) (20) allows the cleavage by CNBr. When the isolated native human peptide was treated with this reagent (see *Materials and Methods*), three peptides could be isolated by HPLC (24). Their amino acid compositions after 24 hr of hydrolysis (Table 2) showed that the total number of amino acid residues predicted is again 76, and the compositions of the peptides CNBr I and CNBr II were exactly as would be predicted from the reported preliminary structure (20). Peptide CNBr III has one residue of glycine less than that predicted from amino acid composition studies (20).

Sequence of CNBr II and CNBr III. The sequence of CNBr II and CNBr III (CNBr-I fragment, being the NH_2-terminus, was not sequenced) allowed the unequivocal identification of the COOH-terminal segment of the human peptide directly (Fig. 1). Use of Polybrene in the sequenator cup allowed the sequence of CNBr II to proceed up to Val-52. The position of Met-53 was obtained previously by tryptic mapping (20). Furthermore, analysis of CNBr II showed a GalN-to-GlcN ratio of about 2:1. Because no Asn-Xaa-Thr/Ser sequence was present within this segment (Fig. 1), an *N*-glycosidic linkage was excluded (27). The only other type of glycosidic linkage known would be an *O*-glycosylation. Because only one Thr is in this peptide and no Ser, we propose that Thr at position 45 is glycosylated. In accordance with this hypothesis, *O*-glycosylation on either Ser or Thr usually occurs in a proline-rich region and contains galactosamine as the major hexosamine (28). This is exactly the situation in peptide CNBr II. Furthermore, a similar conclusion was reached for the porcine homologue (23). However, positive proof of such glycosylation would require the isolation of the putative Thr-45 carbohydrate entity after exhaustive enzymatic digestion.

The automatic sequence of CNBr III also was performed, and it proceeded smoothly up to the COOH-terminal glutamine (Fig. 1). Comparison of the sequence results and those from the amino acid composition shows that all residues are accounted for. Furthermore, at position 65 (cycle 12 in CNBr III se-

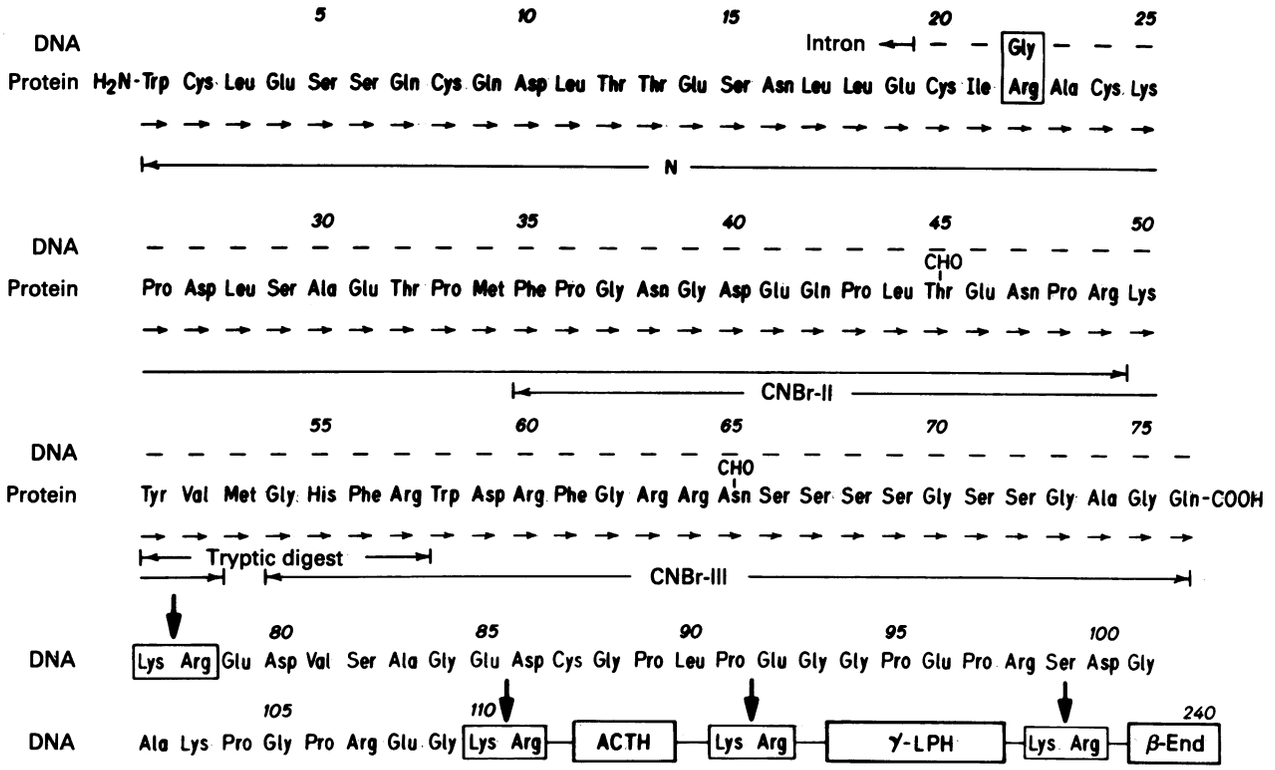

FIG. 1. Comparisons of human genomic DNA sequence of pro-opiomelanocortin (16) with that obtained in this study from protein sequencing of the isolated human NH_2-terminal peptide. At residue 19 of the DNA sequence, an intron site was reported (16). Only one difference between DNA and this protein sequence was found—at residue 22. ----, Identity of sequence; →, direct protein sequence; CHO, proposed glycosylation site; tryptic digest, peptide confirmed previously in a number of fragments (19, 20). ←N→, ←CNBr II→, and ←CNBr III→, number of residues sequenced in the native NH_2-terminal, CNBr II, and CNBr III peptides, respectively; ↓, preferred sites of cleavage in human pro-opiomelanocortin, based on this work, the genomic DNA sequence (16), and known human pituitary contents (1). β-End, β-endorphin.

quence), no residue could be identified (24). A similar situation was observed upon sequencing asparagine-linked carbohydrate residues in mouse nerve growth factor γ-subunit (29). The explanation given was the insolubility of phenylthiohydantoin-Asn-sugar moities in the sequenator extraction solvent (butyl chloride). Therefore, this confirms our previous designation of Asn-65 as being one of the glycosylation sites in the human NH_2-terminal peptide (20). Furthermore, the GlcN-to-GalN ratio was found to be close to 3:1 (Table 2). This is similar to the ratio expected from the sequence of an asparagine-linked oligosaccharide chain in bovine and ovine luteinizing hormone (30). The absence of Gly-66 and the sequence inversion between Gly-72 and Ser-75 (20) compared to the bovine homologue as predicted from the human genomic DNA sequence (16) is now confirmed. The error in the previously proposed sequence (20) can be explained because it was based solely on amino acid composition and homology with the bovine sequence; our Gly value was 3.5 and erroneously taken as 4, rather than 3.0, in tryptic peptide T14 (20). In fact, this is the danger that one runs if sequence is based only on homology in amino acid composition because sequence inversions cannot be determined. Nevertheless, the majority of the preliminary sequence reported is now confirmed.

Finally, comparison between the complete 76-residue peptide sequence and that predicted from the human genomic DNA shows only one variation at residue 22 (Fig. 1). Our results complete the sequence of the first 19 amino acid residues missing from the human genomic DNA studies (16).

DISCUSSION

The isolated human NH_2-terminal peptide is obtained in good yield (15 mg/250 glands) and, hence, represents an important pituitary peptide. From Fig. 1, one can see that the release of such a peptide would involve a cleavage at the pair of basic amino acid residues Lys^{77}-Arg^{78}. From the structure of known prosecretory proteins, the remarkable fact emerges that all of the proproteins studied so far contain paired basic residues at the sites of cleavage, and that arginine rather than lysine seems to be preferred on the carboxyl side of the pair (31). One exception to this is the Lys-Lys pair in β-LPH located just before the β-MSH sequence (32). However, much work now supports the view that this bond could be cleaved only in the pars intermedia during the maturation of the product. In the human pituitary gland, which lacks a pars intermedia, the only products known to be secreted are now the NH_2-terminal peptide (containing residues 1–76), ACTH, β-LPH, γ-LPH, and β-endorphin. Thus, it is clear that in the human pars distalis, the only pair of basic amino acids cleaved in pro-opiomelanocortin is Lys-Arg (Fig. 1). This remarkable selectivity could well speak for an as yet uncharacterized but highly specific maturation enzyme(s).

From the data presented in this paper, only one variation is seen between the sequence of the peptide isolated and that predicted from the sequence by Chang *et al.* (16) of a genomic DNA—namely, Arg-22 instead of Gly-22. This difference could be due to replication errors involved in the technique of recombinant DNA or to uniqueness of the sequenced fetal DNA (16). The absence of any traces of Gly at that residue (24) would argue against its being a polymorphic variation because at least 250 glands were used to purify the NH_2-terminal peptide.

The highly conserved γ-MSH sequence (19, 20, 23) is flanked on both sides by glycosylation sites (Fig. 1). The function of the latter is yet to be adequately explained. This is especially im-

Table 2. CNBr peptides

Amino Acid	CNBr I (1–34)	CNBr II (35–53)	CNBr III (54–76)
Asx	3.10(3)	2.93(3)	2.50(2)
Thr	2.94(3)	0.99(1)	—
Ser	3.65(4)	—	5.81(6)
Glx	6.21(6)	3.23(3)	1.15(1)
Pro	1.78(2)	3.17(3)	—
Gly	—	2.25(2)	4.93(5)
Ala	2.39(2)	—	0.97(1)
Cys	ND(4)	—	—
Val	—	1.11(1)	—
Met	+(1)*	+(1)*	—
Ile	0.88(1)	—	—
Leu	4.95(5)	1.06(1)	—
Tyr	—	0.92(1)	—
Phe	—	1.04(1)	2.00(2)
His	—	—	1.01(1)
Lys	1.24(1)	0.67(1)	—
Trp	ND(1)†	—	1.43(1)
Arg	0.95(1)	1.36(1)	3.93(4)
GlcN	—	0.46	0.83
GalN	—	0.85	0.35
Total	34	19	23

Amino acid composition of CNBr peptides obtained from the isolated native human NH_2-terminal peptide. The numbers in parenthesis beside each CNBr denote the peptide it represents (see Fig. 1). It is seen that although peptide CNBr I is not glycosylated, CNBr II and CNBr III are but have opposite GlcN-to-GalN ratios, possibly representing *O*- and *N*-glycosylation chains respectively (see text). ND, not determined but confirmed by sequence.

* Determined as presence of homoserine and homoserine lactone.

† In this peptide the Trp was completely destroyed upon hydrolysis.

portant because, although *N*-acetylgalactosamine is the linkage monosaccharide commonly present in *O*-glycosidically linked carbohydrate units, its presence in the *N*-glycosidic carbohydrates has been discovered only recently in the pituitary luteinizing hormone (30). This seems to be the case for the Asn-65 linkage (Table 2). However, the nature of the carbohydrate structure will have to await complete oligosaccharide characterization. Such carbohydrate structures could well be involved in guiding the maturation of prosecretory proteins (10, 33, 34).

In view of the finding that the isolated glycosylated 76-amino acid NH_2-terminal peptide migrates in the presence of Na-$DodSO_4$ with an apparent molecular weight of about 18,000 (19, 20), it is tempting to speculate whether a peptide of similar COOH-terminal length (74 amino acids in rat) (18) represents the 16,000 molecular weight variant in mouse AtT-20 tumor cells (4, 7, 10) and in rat pars intermedia (5, 11–13). Because all of these pulse–chase experiments relied on either NH_2-terminal sequence analysis or peptide mapping, or both, that possibility remains to be clarified. Furthermore, the length of human, bovine, rat, and porcine (unpublished data) homologous peptides would vary from 76, 77, 74, and 80 residues, respectively, and, hence, interspecies sequence deletions or additions seem to occur in this region of the molecule. Nevertheless, in all deletions observed, the structure Asn-Xaa-Ser remains there, except that Xaa can either be a Gly or Ser, possibly to ensure glycosylation at that site. Furthermore, from all the sequences known (15–18, 20–23; unpublished data) the Thr-45 is also invariant and also could represent another potential glycosylation site in all these species.

The peptide whose sequence was determined in this study was clearly shown to be 76 amino acids long. In a previous preliminary study (20), a 103-amino acid peptide was proposed, based on amino acid analysis of tryptic fragments, homology to bovine cDNA sequence (15), and NH_2-terminal sequence analysis of the first 42 residues. Although two tryptic peptides, T15 and T16, were found to contain an extension to the 76 residues (20), they were isolated only in minor quantities. Therefore, the possibility remains that this peptide is slightly contaminated by a larger homologous molecule. However, based on genomic DNA sequence studies, the predicted length would be 109 (ref. 16; Fig. 1). Preliminary two-dimensional Na$DodSO_4$ slab gel electrophoresis studies indicated the possible presence of one major band (pI 5.3) and of two extra bands of higher apparent molecular weights and pIs of 5.0 and 4.9, therefore pointing out a possible minor heterogeneity in our preparation (unpublished observations).

Recently, considerable interest in the NH_2-terminal peptide of pro-opiomelanocortin was created when it was shown that a tryptic peptide of the mouse 16,000 molecular weight variant (35) and a γ_3-MSH synthetic peptide could act in synergism with ACTH to activate the release of glucocorticoids (36). In another study, the 80-residue glycosylated NH_2-terminal peptide isolated from porcine pituitaries (23; unpublished data) also was shown to stimulate the release of aldosterone from a human adrenal adenoma *in vitro* (37). In the latter study, it also was shown to be more potent than γ_3-MSH, ACTH, or angiotensin II in stimulating aldosterone release.

The same aldosterone-stimulating activity was observed also with the human 76-residue peptide reported in this study (24). This observation points out the possible involvement of a pituitary factor, similar to the isolated NH_2-terminal peptide, in aldosterone control. Such a hypothesis seems to agree with a recent report (38) which shows that an aldosterone-stimulating factor could be purified from human urine, and with an earlier finding (39) that plasma aldosterone release in nephrectomized dogs does not depend solely on ACTH but that another pituitary factor could be involved. Therefore, much work is necessary before we can understand the biological function of the human NH_2-terminal peptide isolated, which possesses a potent *in vitro* aldosterone-stimulating activity.

Note Added in Proof. The missing fragment 79–108 (Fig. 1) was isolated, and the sequence was determined; the fragment was shown to have an identical sequence to that predicted from DNA study (16) except that Gly-109 is missing, thereby giving a peptide of 30 amino acid residues, possibly amidated at the glutamic acid COOH-terminus.

We thank Mr. Jim Rochemont for his expert technical assistance on the amino acid analyzer and phenylthiohydantoin derivative separation by HPLC. We also wish to thank Miss J. Hamelin and Mr. G. De Serres for excellent technical assistance. Dr. C. Lazure is to be thanked for critically reading the manuscript and Mrs. D. Marcil for typing the manuscript. This work was supported by a Program Grant from the Medical Research Council of Canada, a grant from the National Institutes of Health, and a grant from the National Cancer Institute of Canada.

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